



TITLE:

Photothermal ablation of tumor cells using a single-walled carbon nanotube-peptide composite.

AUTHOR(S):

Hashida, Yasuhiko; Tanaka, Hironori; Zhou, Shuwen;
Kawakami, Shigeru; Yamashita, Fumiyoshi; Murakami,
Tatsuya; Umeyama, Tomokazu; Imahori, Hiroshi; Hashida,
Mitsuru

CITATION:

Hashida, Yasuhiko ...[et al]. Photothermal ablation of tumor cells using a single-walled carbon nanotube-peptide composite.. Journal of controlled release 2014, 173: 59-66

ISSUE DATE:

2014-01-10

URL:

<http://hdl.handle.net/2433/189759>

RIGHT:

© 2013 Elsevier B.V.; This is not the published version. Please cite only the published version.; この論文は出版社版ではありません。引用の際には出版社版をご確認ご利用ください。

Photothermal ablation of tumor cells using a single-walled carbon nanotubes-peptide composite

Yasuhiko Hashida,^a Hironori Tanaka,^b Shuwen Zhou,^b Shigeru Kawakami,^b Fumiyoshi Yamashita,^b Tatsuya Murakami,^a Tomokazu Umeyama,^c Hiroshi Imahori,^{a,c} and Mitsuru Hashida^{a,b,*}

^aInstitute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Kyoto, Japan

^bDepartment of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

^cDepartment of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto, Japan

* Corresponding author at: Department of Drug Delivery Research, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan.

Tel: +81 75 753 4525; fax: +81 75 753 4575.

E-mail addresses: hashidam@pharm.kyoto-u.ac.jp (M. Hashida)

Abstract

Single-walled carbon nanotubes (SWCNTs) are known to have great potential for biomedical applications such as photothermal ablation of tumor cells in combination with near-infrared (NIR) irradiation. In this study, the photothermal activity of a novel SWCNTs composite with a designed peptide having a repeated structure of H-(-Lys-Phe-Lys-Ala-)₇-OH [(KFKA)₇] against tumor cells was evaluated *in vitro* and *in vivo*. The SWCNTs-(KFKA)₇ composite demonstrated high aqueous dispersibility that enabled SWCNTs to be used in tumor ablation. The NIR irradiation of SWCNTs-(KFKA)₇ solution resulted in a rapid temperature increase dependent on the SWCNTs concentration up to 50 µg/ml. Three minutes of NIR irradiation of a colon26 or HepG2 cell culture incubated with SWCNTs-(KFKA)₇ resulted in remarkable cell damage, while that by single treatment with SWCNTs-(KFKA)₇ or NIR irradiation alone was moderate. Intratumoral injection of SWCNTs-(KFKA)₇ solution followed by NIR irradiation resulted in a rapid increase of the temperature to 43 °C in the subcutaneously inoculated colon26 tumor based on thermographic observation and remarkable suppression of tumor growth compared with treatment with only SWCNTs-(KFKA)₇ injection alone or NIR irradiation alone. These results suggest the great potential of a SWCNTs-peptide composite for use in photothermal cancer therapy.

Keywords

Single-walled carbon nanotube

Single-walled carbon nanotube-peptide composite

Near infrared laser irradiation

Tumor ablation

Photothermal cancer therapy

Aqueous dispersibility

1. Introduction

Carbon nanotubes (CNTs) have been widely studied from the viewpoint of potential medical applications because of their unique and useful physical, chemical, electrical, and mechanical properties [1, 2]. Attempts have been made, for example, to utilize the intrinsic hyperthermic property of CNTs induced by near-infrared (NIR) irradiation for the photothermal ablation of cancer cells [3]. In general, however, studies on the use of CNTs in biological, medical, and pharmaceutical applications have not advanced due to the high hydrophobicity of CNTs, which makes them incompatible with living organisms or biological settings. To improve the poor dispersibility of CNTs into aqueous media, we have developed a novel composite material of single-walled carbon nanotubes (SWCNTs) with artificially designed peptides and evaluated its chemical and physicochemical characteristics with an aim toward biomedical application [4]. The formation of the composite of SWCNTs with peptide (SWCNTs–peptide) was confirmed by atomic force microscopy, transmission electron microscopy, and molecular modeling [4].

In an ongoing series of investigations, we have evaluated the utility of SWCNTs–peptide in various aspects of biomedical application including tumor ablation. Near-infrared light (NIR) at a region of 700-900 nm in wavelength is known to be relatively harmless to the body even though it penetrates deep into the tissue [5]. The electromagnetic wave in this region shows minimal absorption by media such as hemoglobin (absorption <650 nm) and water (absorption >900 nm) [6] whereas SWCNTs can effectively absorb NIR and convert its energy into heat [3]. Because of this feature, SWCNTs would seem to be promising for use in noninvasive photothermal cancer therapy under NIR irradiation [7, 8, 9].

Among tested peptides in previous report, composite with H-(-Lys-Phe-Lys-Ala-)₇-OH [(KFKA)₇] showed satisfactory dispersibility and stability in water for injection [4]. Expected binding to tumor tissue based on electrostatic interaction [10] and possibility of introducing various functions such as controlled release of anticancer agents [4, 10] further encourage the application of (KFKA)₇ in cancer ablation. Thus the (KFKA)₇ peptide was employed to solubilize and thereby improve the therapeutic effects of SWCNT, and the prepared SWCNTs-(KFKA)₇ composite was evaluated for its photothermal characteristics and antitumor activity in combination with NIR laser irradiation in this study.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments were carried out in accordance with Guide for the Care and Use of Laboratory Animals by the U.S. National Institutes of Health (Bethesda, MD) and the Guidelines for Animal Experiments of Kyoto University (Kyoto Japan). The protocol was approved by the Kyoto University Animal Experimentation Committee (iCeMS Kyo-7-4). All surgery was performed under sodium pentobarbital anesthesia.

2.2. Materials

Purified SWCNTs (HiPco; Lot No. P0343) were purchased from Carbon Nanotechnologies (Houston, TX). The (KFKA)₇ peptide shown in Fig. 1 was designed by expecting self-assembled wrapping of SWCNTs [4] and synthesized by GL Biochem (Shanghai, China) with more than 90% purity. Triton X-100 was purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), and Hanks' balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum was purchased from MP Biomedicals (Irvine, CA). Other chemicals were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemicals (Osaka, Japan).

2.3. Preparation of SWCNTs solution

Dispersion of SWCNTs was prepared by sonicating SWCNTs with (KFKA)₇ peptide in aqueous media. One milligram of SWCNTs and 10 mg of (KFKA)₇ peptide were weighed and put into a test tube. Then 5 ml of saline or dextrose solution was added to the test tube and sonication was performed for 1 hour with an ultrasonic disruptor UD-201 (TOMY Digital Biology, Tokyo, Japan) on ice.

2.4. Quantification and size determination of SWCNTs in the solution

The concentration of SWCNTs in the solution was determined from the optical absorbance at 808 nm according to the previous report [3]. An absorptive coefficient of $A_{1\text{mg/ml}} = 40.3$ was obtained from the calibration line of the SWCNTs suspension (0-25 μg SWCNTs/ml) prepared with Triton X-100 [4]. The length of SWCNTs was estimated from atomic force microscopic (AFM) image. AFM observation was performed for SWCNTs in the solution using an MFP-3D-SA atomic force microscope (Asylum Technology, Santa Barbara, CA) in AC mode. AC200-TS microcantilevers (Olympus, Tokyo, Japan) with a force constant of $k =$

9 N·m⁻¹ and a nominal tip radius of less than 10 nm were used. Measurements were performed in air. The size of SWCNT in AFM image was measured using an image analysis software, Image J (Ver. 1.47, <http://rsbweb.nih.gov/ij/>).

2.5. Photothermal characteristics of SWCNTs solution with NIR irradiation

The photothermal characteristics of the SWCNTs-(KFKA)₇ composite with NIR laser irradiation was evaluated by continuous temperature monitoring of its aqueous solution. One milliliter of SWCNTs solution supplemented with (KFKA)₇ peptide (0-100 µg SWCNTs/ml) in a vial with a diameter of 1.6 cm and cross-section of 2.0 cm² was irradiated with an NIR laser of 1.2 W (808 nm) (Femtosecond Titanium Sapphire laser Chameleon-RF; Coherent, Santa Clara, CA) over an exposure area of 0.2 cm² (6 W/cm²). During irradiation, the SWCNTs solution was stirred with a magnetic stirrer and the temperature of the solution was measured each second using a fiber optic temperature sensor Reflex (Neoptix, QC, Canada).

2.6. Cell culture

The murine rectum carcinoma cell line (colon26) and human hepatocellular carcinoma cell line (HepG2) were cultured in RPMI 1640 medium and DMEM, respectively, under 5% CO₂ at 37 °C. The culture medium was supplemented with 10% fetal bovine serum, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin.

2.7. Cytotoxicity assay for (KFKA)₇ peptide

The cytotoxicity of the (KFKA)₇ peptide was evaluated by measuring the activities of lactate dehydrogenase (LDH) released from damaged cells to the medium [11, 12]. Colon26 cells and HepG2 cells were seeded in 24-well plates (1 × 10⁵ cells/well) and incubated overnight. Then, the culture medium was removed and 400 µl of medium containing 0-100 µM of (KFKA)₇ peptide and 1% FBS were added. After 6 hours of incubation, the plates were centrifuged at 250 × g for 10 min at 4 °C and the activity of LDH in the supernatant was measured with an LDH Cytotoxicity Detection Kit (Takara Bio, Shiga, Japan). As a positive control, cells were treated with the medium containing 1% Triton X-100 for 6 hours and the amount of released LDH was measured in the same way. The 50% inhibitory concentration (IC₅₀) values of (KFKA)₇ peptide against both cell lines were calculated by fitting to a logistic model function.

2.8. *In vitro* evaluation of cell death induced by SWCNTs-(KFKA)₇ with NIR irradiation

The damage to tumor cells induced by thermal ablation with SWCNTs-(KFKA)₇ and NIR irradiation was evaluated *in vitro*. Colon26 cells and HepG2 cells (2×10^5 cells/500 μ l) were seeded in an 8-well chambered coverglass (Asahi Glass, Tokyo, Japan) and incubated overnight. After changing the culture medium, 1.5 μ l and 5 μ l of SWCNTs solution (200 μ g SWCNTs/ml) was added to the 400 μ l of culture medium of colon26 cells and HepG2 with final concentrations of 0.75 μ g/ml and 2.5 μ g/ml, respectively. After 2 hours of incubation, the wells were exposed to irradiation with an 808 nm NIR laser for 3 min at 1.2 W, collected in a 1.5 ml tube, and stained with a Live-Dead cell staining kit (Biovision, Mountain View, CA).

The fluorescence microscopic observation was performed using a Biozero Bz-8000 (Keyence, Osaka, Japan) with Ex/Em = 470/535 nm (Live-Dye fluorescing green) and Ex/Em = 540/605 nm (propidium iodide fluorescing red), respectively. Confocal microscopy was carried out with A1RMP (Nikon, Tokyo, Japan) with Ex/Em = 488/525 nm (green) and Ex/Em = 562/595 nm (red), respectively. For flow cytometric analysis, cells were stained with propidium iodide of a Live-Dead cell staining kit and the number of labeled cells was analyzed by a FACSCant II (BD biosciences, San Jose, CA) with Ex/Em = 488/585 nm.

2.9. *In vivo* monitoring of temperature increase in the tumor induced by SWCNT-(KFKA)₇ with NIR irradiation

To the flank of 5-week-old female BALB/c mice, 100 μ l of the colon26 cell suspension at 5×10^6 cells/ml in HBSS was subcutaneously injected. After the implanted tumor grew to a mean diameter of 5 mm, SWCNT-(KFKA)₇ composite [1 μ g of SWCNTs and 10 μ g (KFKA)₇] in 100 μ l of 5% dextrose solution was injected into the tumor and, 24 hours later, NIR laser irradiation was carried out for 30 s with an 808 nm NIR laser at 1.2 W (6 W/cm²) under sodium pentobarbital anesthesia (Nacalai Tesque, Kyoto, Japan). The change of temperature in the tumor was monitored using an InfReC Thermography R300 (NEC Avio Infrared Technologies, Tokyo, Japan).

2.10. *In vivo* therapeutic activity of SWCNT- (KFKA)₇ composite with NIR laser irradiation

The therapeutic effect of ablation with SWCNTs-(KFKA)₇ composite and NIR

laser irradiation was evaluated by monitoring the growth inhibition of the tumor inoculated in mice. To the flank of 5-week-old female BALB/c mice, 100 μ l of the colon26 cell suspension at 5×10^6 cells/ml in HBSS was subcutaneously injected. To measure the tumor growth, the tumor volume (V) was estimated with the following equation:

$$V = (4/3) \cdot \pi \cdot (x/2) \cdot (y/2) \cdot (z/2)$$

In this equation, x, y, z, and π represent the length, width, and height of the tumor and circular constant, respectively. When the tumor volumes reached to about 100 mm³, mice were randomized into 7 groups and 50 μ l of SWCNTs suspension containing 0 (2 groups), 1 (one group), 5 (2 groups), or 10 μ g (2 groups) of SWCNTs was injected into the tumor. At 24, 48, and 72 hours after the SWCNTs injection, the mice of four groups with different doses of SWCNTs were anesthetized with sodium pentobarbital (Nacalai Tesque, Kyoto, Japan) and the tumor mass was irradiated with an NIR laser at 808 nm. The NIR laser treatment consisted of three rounds of 60-sec illumination at 1.2 W for 0.2 mm² (6 W/cm²) with 30-sec-intervals. After NIR irradiation, the tumor sizes were measured every 2-5 days for 34 days. Results are shown as the means and S.D. (n = 5) and statistical analysis was carried out with Tukey-Kramer multiple comparison test.

2.11. Retention and localization of SWCNT-(KFKA)₇ composite in the tumor

Localization of SWCNTs-(KFKA)₇ composite in the tumor tissue was examined with histological observation. To the solid tumor of colon26 implanted on the flank of 5-week-old female BALB/c mice, 50 μ l of SWCNTs solution containing 10 μ g of SWCNTs was injected. At 24 and 72 hours after injection, the tumors were excised, fixed in 4% paraformaldehyde, and embedded in paraffin. Then 5 μ m-sections were made using a microtome and stained with hematoxylin and eosin for histological observation by photomicrography (Biozero Bz-8000; Keyence, Osaka, Japan).

3. Results

3.1 Preparation and characterization of SWCNTs-(KFKA)₇ solution

SWCNTs-(KFKA)₇ solution has good dispersibility and stability at a room temperature. The AFM image of SWCNTs-(KFKA)₇ composites shown in Fig.

2A demonstrates that they individually suspended in water in a single tube form and average length of SWCNTs was estimated to be 280 nm from 208 counts of SWCNTs.

3.2. Temperature increase of SWCNTs-(KFKA)₇ solution induced by NIR irradiation

The photothermal characteristics of SWCNTs dispersed with (KFKA)₇ peptide in water were evaluated. The temperature of 1 ml of SWCNTs solution (0-100 µg/ml) rose with the period of NIR irradiation and also with the concentration of SWCNTs, as shown in Fig. 3A. At 300 s after the start of irradiation, the highest increase of temperature, i.e., more than 20 °C, was observed for the SWCNTs solution at a concentration of 100 µg/ml while NIR irradiation to saline showed only an increase of 3 °C. These results suggest that NIR laser irradiation to the SWCNTs-(KFKA)₇ composite in water resulted in heat generation with relatively high efficiency. The relationship between the photothermal effect and SWCNTs concentration was further analyzed based on the initial rates of temperature increase estimated from the time course, and the results are plotted against the SWCNTs concentration in Fig. 3B. In this plot, the rate of temperature increase can be seen to rise with increasing concentration up to 50 µg/ml, and then to reach a relative plateau.

3.3. Evaluation of the direct cytotoxicity of (KFKA)₇ on cultured cells

The cytotoxicity of the (KFKA)₇ peptide itself dissolved in culture medium was evaluated by LDH assay. Colon26 and HepG2 cells were exposed to the (KFKA)₇ peptide at different concentrations for 6 hours and LDH assay was carried out. As shown in Fig. 4, the (KFKA)₇ peptide showed relatively low toxicity with less than 20% cell damage under a concentration of 10 µg/ml for colon26 cells and 33 µg/ml for HepG2 cells, respectively. At concentrations higher than this, the leakage of LDH increased with concentration, and the 50% inhibitory concentration (IC₅₀) values of (KFKA)₇ peptide against colon26 and HepG2 were estimated to be 28.6 µg/ml and 131 µg/ml, respectively.

3.4. In vitro evaluation of cell damage induced by SWCNTs-(KFKA)₇ with NIR irradiation

To evaluate the lethal effect of SWCNTs-(KFKA)₇ with NIR laser irradiation, the damage to colon26 (A, B, C, D) and HepG2 cells (E, F, G, H) caused by these treatments alone or in combination was assessed by fluorescence microscopy

observation and the results are shown in Fig. 5. The cell samples without any treatment show mostly living cells indicated by green fluorescence (A, E). In the samples treated by SWCNTs-(KFKA)₇ at final concentrations of 0.75 µg/ml for colon26 and 2.5 µg/ml for HepG2 cells, respectively, and NIR laser irradiation, a large number of dead cells were observed in both cell lines as yellow-red reflecting overlay of red and green fluorescence (D, H). Samples having treatment with only SWCNTs-peptide (B, F) or NIR irradiation (C, G) exhibited moderate cell damage.

Cell samples treated by SWCNTs-(KFKA)₇ and NIR laser irradiation were also examined by confocal microscopy for colon26 (I) and HepG2 cells (J), respectively. Cell damage in dead cells stained by merge of red and green is exhibited in cell figures.

In order to obtain quantitative information on cell damage, percentages of dead cells stained by red fluorescence against total cell numbers were estimated by flow cytometry and results are shown in Fig. 6 for colon26 (A, B, C, D) and HepG2 cells (E, F, G, H). While cells without any treatments mostly distributed in the area without red fluorescence staining, cells having combination treatment of SWCNTs-(KFKA)₇ and NIR laser irradiation demonstrated cell death with 55.8 % for colon 26 (D) and 86.7 % for HepG2 (H), respectively. Treatment with only SWCNTs-(KFKA)₇ alone (B, F) or NIR irradiation alone (C, G) exhibits moderate damage.

These results suggest that the combination of SWCNTs-(KFKA)₇ and NIR irradiation is required in order to achieve a lethal effect on cells.

3.5. In vivo photothermal anticancer activity in the combination of SWCNTs-(KFKA)₇ and NIR laser irradiation

The in vivo ablation effects of SWCNTs-(KFKA)₇ with NIR irradiation on the subcutaneously implanted colon26 tumor were evaluated by monitoring the local temperature increase and the tumor-growth inhibition.

As shown in Fig. 7, the temperature of the tumor tissues treated with SWCNTs-(KFKA)₇ followed by local NIR laser irradiation increased rapidly and reached approximately 43 °C at 30 s after the start of irradiation. On the other hand, the tumor tissues receiving irradiation without SWCNTs-(KFKA)₇ injection showed only a slight increase of temperature even after 30 s of NIR irradiation.

The in vivo ablation activity of SWCNTs-(KFKA)₇ with NIR irradiation was evaluated by monitoring tumor growth after the treatment, and the results are shown in Fig. 8. In the control group without any treatment and the groups with only irradiation or SWCNTs-(KFKA)₇ injection, the tumor grew rapidly, with the volume reaching 1500-2000 mm³ at 34 days after implantation. No statistically

significant differences ($P > 0.05$) in the tumor growth rate or the final tumor size were observed among these groups, suggesting that tumor growth was not affected by either laser irradiation alone or SWCNTs-(KFKA)₇ injection alone. In contrast, statistically significant suppression ($P > 0.05$) of tumor growth was observed in the groups treated with SWCNTs-(KFKA)₇ plus NIR irradiation, although a dose-dependent attenuation in growth was not observed in these groups. Thus, injection of even 1 μg SWCNTs with NIR irradiation was shown to be effective to realize a therapeutic effect against colon26 tumors. On the other hand, none of the groups receiving combination treatment with SWCNTs-(KFKA)₇ and NIR irradiation achieved a complete reduction of the tumor, since tumor was still detected in some of the cases of each group at 34 days after tumor implantation.

Localization of SWCNTs-(KFKA)₇ in the tumor at 24 (A) and 72 hours (B) after intratumoral injection was examined by histological observation and results are shown in Fig. 9 (A, B). In the tumor, injected SWCNTs-(KFKA)₇ was considerably deposited along the space made by needle insertion and pour of injection solution suggesting sustained localization but limited dispersion in the tumor.

4. Discussion

In previous study [4], we have designed seven types of peptides with an aim toward biomedical application of SWCNTs. The peptides were designed to form β -sheet structure that would be suitable for wrapping SWCNTs. The possibility of introducing various functions to SWCNT-peptide was also demonstrated by several methods, such as introduction of special amino acids, chemical modification, and additional complex formation based on electrostatic interaction. Among tested, SWCNTs-(KFKA)₇ composite with satisfactory dispersibility and stability in water was evaluated for its photothermal activity in this study.

In the experiment determining heat generation efficiency of SWCNTs-(KFKA)₇ and NIR irradiation, an apparent maximum temperature increase rate of 0.11 $^{\circ}\text{C}/\text{s}$ was observed (Fig. 3B). In this experiment, the maximum rate of temperature increase $[(dT/dt)_{\text{max}}]$, under the assumption that all irradiated energy would turn to heat, was estimated from the following equation:

$$(dT/dt)_{\text{max}} = (\text{irradiation energy})/(\text{standard calorie})/(\text{volume of water})$$

where the volume of a sample was 1 ml, the irradiation energy was 1.2 W (1.2 J/s), and a standard calorie is 1 cal = 4.185 J. This equation yielded a $(dT/dt)_{\text{max}}$ value of 0.286 $^{\circ}\text{C}/\text{s}$. While the optical absorbance of SWCNTs at 808 nm with a

0.5 cm light-pass length is around 1 for a solution of 50 $\mu\text{g/ml}$, the efficiency of the energy transfer from light to heat as well as the leaking of generated heat from the SWCNTs solution to the outside system would also have contributed to this result. In consideration of the above, it appeared that a considerable amount of NIR energy was absorbed by SWCNTs at a concentration above 50 $\mu\text{g/ml}$ and was converted to heat with rather good efficiency under the present conditions.

The results of the cytotoxicity evaluation for (KFKA)₇ suggest that this peptide had moderate toxicity on cultured cells (Fig. 4). Since the (KFKA)₇ peptide has 14 lysyl residues per 28 amino acids in one molecule, it should be highly cationic at a neutral pH. Like other polycations, (KFKA)₇ would show some cytotoxicity by perturbing or damaging cell membranes through neutralization of the negative charges within them [13, 14]. However, its cytotoxicity is negligible at lower concentrations.

On cultured colon26 and HepG2 cells, SWCNTs-(KFKA)₇ with NIR irradiation effectively induced cell death at concentrations of 0.75 $\mu\text{g/ml}$ and 2.5 $\mu\text{g/ml}$ of SWCNTs (7.5 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$ as (KFKA)₇), respectively. Under these conditions, the concentrations of free (KFKA)₇ are considerably lower than the IC₅₀ values of (KFKA)₇ peptide itself against colon26 (28.6 $\mu\text{g/ml}$) and HepG2 cells (131 $\mu\text{g/ml}$), regardless of the stoichiometry of SWCNTs and (KFKA)₇ composite formation [4]. Therefore, the cytotoxic effects of SWCNTs-(KFKA)₇ with NIR irradiation should be attributed to the ablation activity of their combination but not to the cytotoxicity of (KFKA)₇ itself, although an electrostatic interaction between the cell surface and polycationic SWCNTs-(KFKA)₇ might play a role in the ablation in part. On the other hand, we recently reported that semiconducting SWCNTs generates reactive oxygen species by NIR irradiation [15] so that participation of the photodynamic effect of SWCNTs might be considered in addition to the photothermal effect.

In the in vivo ablation experiment, colon26 tumors subjected to intratumoral injection of 1 μg SWCNTs-(KFKA)₇ showed rapid elevation of local temperature to approximately 43 °C during 30 s NIR laser irradiation, which is generally considered an effective temperature for hyperthermia therapy [16]. In accordance with this, injection of SWCNTs-(KFKA)₇ suspension and NIR irradiation demonstrated significant inhibition of colon26 growth, but did not achieve complete eradication of the tumors. In this experiment, 1-10 μg SWCNTs-(KFKA)₇ was injected into a colon26 tumor with a volume of about 100 mm³, and thus the estimated concentration of SWCNTs should be higher than 10 $\mu\text{g/g}$ in all cases. Although this concentration fairly exceeds the concentration of SWCNTs-(KFKA)₇ that showed significant cell damage in the in vitro experiment (0.75 $\mu\text{g/ml}$), the therapeutic effects were still limited. Histological observation shown in Fig. 9 demonstrates that injected SWCNTs-(KFKA)₇ was

considerably deposited along the space made by needle insertion and pour of injection solution, suggesting limited dispersion of SWCNTs-(KFKA)₇. Because SWCNTs-(KFKA)₇ has a needle shape with an average length of around 280nm (Fig. 2), it would be reasonable to be kept around the injected area. A cooling effect due to blood circulation would account for some of the limitation of the ablation effect, and heterogeneity of the cell composition or the anatomical/histological structure of the tumor could also have undermined the therapeutic effect. The small numbers of tumor cells surviving even after the ablation treatment would have contributed to the slow but continuous growth of tumor. Therefore, in order to achieve success in photothermal therapy using SWCNTs and NIR irradiation, it should be necessary to improve tissue dispersion profiles of SWCNTs in the tumor and/or to change the protocol of NIR irradiation to such as separate and decentralized injection and repetitious and longer irradiation.

As discussed before, the potential of CNTs in biomedical applications is fully dependent on their compatibility with biological circumstances, and ease of access is also very crucial. To improve the poor dispersibility of CNTs into aqueous media, various methods have been developed [9], such as introduction of hydrophilic functional groups by chemical modification [17, 18, 19, 20], micellization using various surfactants [21, 22], and complex formation with diverse macromolecules [23, 24]. However, direct introduction of large numbers of hydrophilic groups with chemical reactions would lead to a loss of the inherent unique physicochemical and spectrophotometric properties of CNTs, because it is mostly accompanied through destruction of the homogeneous molecular structure of CNTs by reactions such as strong oxidation [18, 19]. Micellization of CNTs with a surfactant looks to be a better solution, and many surfactants, such as sodium dodecyl sulfate (SDS) [21] and Triton X-100 [22], have been proposed for this purpose. However, most surfactants damage the cell membrane, and difficulty in the functionalization of CNTs further hampers their application. Formation of a tighter complex between CNTs and amphiphilic polymer via multi-point interaction, on the other hand, may allow functionalization through the introduction of functional molecules to the polymer [9]. In addition to synthetic polymers, biomolecules such as nucleotides [23], proteins [24], saccharides, and phospholipids have been reported for this purpose. In particular, PEGylated phospholipids achieve not only dispersion of CNTs but also functionalization via covalent attachment of the functional moiety [20, 25], but in most cases, they only can introduce properties related to their original characteristics and lack wide applicability for the introduction of various functions to CNTs [9, 25, 26].

The SWCNTs-peptide composite has an advantage compared with other CNT materials because its physicochemical and/or biological properties are comparable to those of peptides and proteins in general due to its peptide-wrapped surface. Thus, most of the conventional methodologies presently utilized in peptide and protein research are applicable to the functionalization of this composite material. The easily-accessible functional groups strewn on the surface of the CNTs enable the composite to act as a multifunctional vehicle via a combination of these techniques. In tumor ablation, the SWCNTs-peptide composite would have wider application potential compared with SWCNTs in other forms [3, 7, 8], and the introduction of targeting or delivery methodologies reported in our previous studies [27, 28, 29] will be further explored in a subsequent paper.

5. Conclusions

In the present investigation, a novel SWCNTs-peptide composite was evaluated for its tumor ablation activity. NIR irradiation to an SWCNTs-(KFKA)₇ solution effectively induced heat generation and significant damage to cultured colon26 and HepG2 cells in vitro. In addition, the combination of injection of SWCNTs-(KFKA)₇ and NIR irradiation to subcutaneous colon26 tumors in mice achieved a significant reduction of tumor size in accordance with an increase in local temperature in the tumor. Thus, application of (KFKA)₇ increased the potential of SWCNTs by improving their aqueous dispersibility in biological media. The possibility of introducing various functions to the SWCNTs-peptide should further expand its potential in photothermal cancer therapy as well as in drug delivery.

Acknowledgements

A part of this research was supported by a Grant-in-Aid for Scientific Research (No. 22780090) from the Japan Society for the Promotion of Science.

References

- [1] Z. Liu, S. Tabakman, K. Welsher, H. Dai, Carbon nanotubes in biology and medicine: In vitro and in vivo detection, imaging and drug delivery, *Nano Res.* 2 (2009) 85-120.
- [2] N.W.S. Kam, M. O'Connell, J.A. Wisdom, H. Dai, Carbon nanotubes as multifunctional biological transporters and near infrared agents for selective cancer cell destruction, *Proc. Natl. Acad. Sci. USA* 102 (2005) 11600-11605.
- [3] P. Chakravarty, R. Marches, N.S. Zimmerman, A.D.E. Swafford, P. Bajaj, I.H. Musselman, P. Pantano, P.K. Draper, E.S. Vitetta, Thermal ablation of tumor cells with antibody functionalized single-walled carbon nanotubes, *Proc. Natl. Acad. Sci. USA* 105 (2008) 8697-8702.
- [4] Y. Hashida, T. Umeyama, J. Mihara, H. Imahori, M. Tsujimoto, S. Isoda, M. Takano, M. Hashida, Development of a novel composite material with carbon nanotubes assisted by self-assembled peptides designed in conjunction with β -sheet formation, *J. Pharm. Sci.* 101 (2012) 3398-3412.
- [5] S.J. Matcher, C.E. Cooper, Absolute quantification of deoxyhaemoglobin concentration in tissue near infrared spectroscopy, *Phys. Med. Biol.* 39 (1994) 1295-312.
- [6] R. Weissleder, A clearer vision for in vivo imaging, *Nat. Biotechnol.* 19 (2001) 316-317.
- [7] S. Ghosh, S. Dutta, E. Gomes, D. Carrol, R. D'Agostino Jr., J. Olson, M. Guthold, W.H. Gmeiner, Increased heating efficiency and selective thermal ablation of malignant tissue with DNA-encased multiwalled carbon nanotubes, *ACS Nano* 3 (2009) 2667-2673.
- [8] A. Burke, X. Ding, R. Singh, R.A. Kraft, N. Levi-Polyachenko, M.N. Rylander, C. Szot, C. Buchanan, J. Whitney, J. Fisher, H.C. Hatcher, R.Jr. D'Agostino, N.D. Kock, P.M. Ajayan, D.L. Carroll, S. Akman, F.M. Torti, S.V. Torti, Long-term survival following a single treatment of kidney tumors with multiwalled carbon nanotubes and near-infrared radiation, *Proc. Natl. Acad. Sci. USA* 106 (2009) 12897-12902.
- [9] J. Yu, F. Jiao, X. Chen, X. Jiang, Z. Peng, D. Zeng, D. Huang, Irradiation-mediated carbon nanotubes' use in cancer therapy, *J. Cancer Res. Ther.* 8 (2012) 348-354.
- [10] T. Nomura, A. Saikawa, S. Morita, T. Sakaeda (ne Kakutani), F. Yamashita, K. Honda, Y. Takakura, M. Hashida, Pharmacokinetic characteristics and therapeutic effects of mitomycin C-dextran conjugates after intratumoural injection, *J. Control. Rel.* 52 (1998) 239-252.
- [11] C. Korzeniewski, D. M. Callewaert, An enzyme-release assay for natural cytotoxicity, *J. Immun. Meth.* 64 (1983) 313-320.

- [12] T. Decker, M.L. Lohmann-Matthes, A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity, *J. Immun. Meth.* 115 (1988) 61-69.
- [13] L.E. Prevette, G.D. Mullen, M.M.B. Holl, Polycation-induced cell membrane permeability does not enhance cellular uptake or expression efficiency of delivered DNA, *Mol. Pharm.* 7 (2010) 870-883.
- [14] A.C. Hunter, S.M. Moghimi, Cationic carriers of genetic material and cell death: A mitochondrial tale, *Biochim. Biophys. Acta* (2010) 1203-1209.
- [15] T. Murakami, H. Nakatsuji, M. Inada, Y. Matoba, T. Umeyama, M. Tsujimoto, S. Isoda, M. Hashida, H. Imahori, Photodynamic and photothermal effects of semiconducting and metallic-enriched single-walled carbon nanotubes, *J. Am. Chem. Soc.* 134 (2012) 17862-17865.
- [16] B. Hildebrandt, P. Wust, O. Ahlers, A. Dieing, G. Sreenivasa, T. Kerner, R. Felix, H. Riess, The cellular and molecular basis of hyperthermia, *Crit. Rev. Oncology/Hematology* 43 (2002) 33-56.
- [17] J. Liu, A.G. Rinzler, H. Dai, J.H. Hafner, R.K. Bradley, P.J. Boul, A. Lu, T. Iverson, K. Shelimov, C.B. Huffman, F. Rodriguez-Macias, Y.-S. Shon, T.R. Lee, D.T. Colbert, R.E. Smalley, Fullerene pipes, *Science* 280 (1998) 1253-1256.
- [18] M. Prato, K. Kostarelos, A. Bianco, Functionalized carbon nanotubes in drug design and discovery, *Acc. Chem. Res.* 41 (2008), 60-68.
- [19] J. Chen, S. Chen, X. Zhao, L.V. Kuznetsova, S. S. Wong, I. Ojima, Functionalized single-wall carbon nanotubes as rationally designed vehicles for tumor-targeted drug delivery, *J. Am. Chem. Soc.* 130 (2008), 16778-16785.
- [20] S.T. Yang, K.A.S. Fernando, J.H. Liu, J. Wang, H.F. Sun, Y. Liu Y, M. Chen, Y. Huang, X. Wang, H. WangH, Y.P. Sun, Covalently PEGylated carbon nanotubes with stealth character in vivo, *Small* 4 (2008) 940-944.
- [21] M.J. O'Connell, S.M. Bachilo, C.B. Huffman, V.C. Moore, M.S. Strano, E.H. Haroz, K.L. Rialon KL, P.J. Boul, W.H. Noon, C. Kittrell, J. Ma, R.H. Hauge, R.B. Weisman, R.E. Smalley, Band gap fluorescence from individual single-walled carbon nanotubes, *Science* 297 (2002) 593-596.
- [22] V.C. Moore, M.S. Strano, E.H. Haroz, R.H. Hauge, R.E. Smalley, J. Schmidt, Y. Talmon, Individually suspended single walled carbon nanotubes in various surfactants, *Nano Lett.* 3 (2003) 1379-1382.
- [23] M. Zheng, A. Jagota, E.D. Semke, B.A. Diner, R.S. McLean, S.R. Lustig, R.E. Richardson, N.G. Tassi, DNA-assisted dispersion and separation of carbon nanotubes, *Nat. Mater.* 2 (2003) 338-242.

- [24] K. Matsuura, T. Saito, T. Okazaki, S. Ohshima, M. Yumura, S. Iijima, Selectivity of water-soluble proteins in single walled carbon nanotube dispersions, *Chem. Phys. Lett.* 429 (2006) 497-502.
- [25] Z. Liu, C. Davis, W. Cai, L. He, X. Chen, H. Dai, Circulation and long-term fate of functionalized, biocompatible single-walled carbon nanotubes in mice probed by Raman spectroscopy, *Proc. Natl. Acad. Sci. USA* 105 (2008) 1410-1415.
- [26] Z. Liu, X. Sun, N. Nakayama-Ratchford, H. Dai, Supramolecular chemistry on water-soluble carbon nanotubes for drug loading and delivery, *ACS Nano* 1 (2007) 50-56.
- [27] M. Hashida, H. Hirabayashi, M. Nishikawa, Y. Takakura, Targeted delivery of drugs and proteins to the liver via receptor-mediated endocytosis, *J. Control. Rel.* 46 (1997) 129-137.
- [28] Y. Higuchi, M. Oka, S. Kawakami, M. Hashida, Mannosylated semiconductor quantum dots for the labeling of macrophage, *J. Control. Rel.* 125 (2008) 131-136.
- [29] K. Un, S. Kawakami, Y. Higuchi, Ryo Suzuki, Kazuo Maruyama, F. Yamashita, M. Hashida, Involvement of activated transcriptional process in the efficient gene transfection using unmodified and mannose-modified bubble lipoplexes with ultrasound exposure. *J. Control. Rel.* 156 (2011) 355-363.

Figure captions

Fig. 1. Chemical structure of (KFKA)₇ peptide.

Fig. 2. AFM image (A) and size distribution (B) for SWCNTs-(KFKA)₇ in solution.

The size distribution is shown with histogram of sizes of 208 SWCNTs-(KFKA)₇ composites obtained from AFM images.

Fig. 3. Heat generation of SWCNTs-(KFKA)₇ with NIR irradiation.

The SWCNTs-(KFKA)₇ suspension was irradiated with an 808 nm NIR laser at 1.2 W and the temperature was measured with a fiber optic temperature sensor (A). The concentrations of SWCNTs were 100 µg/mL (a), 50 µg/mL (b), 30 µg/mL (c), 10 µg/mL (d), 5 µg/mL (e), 1 µg/mL (f), and 0 µg/mL (g). Photothermal activity was also evaluated, with the initial rate of temperature increase (B) being estimated from the temperature curves.

Fig. 4. Cytotoxicity of (KFKA)₇ peptide.

Colon26 (open circle) and HepG2 (filled circle) cells were exposed to (KFKA)₇ peptide for 6 hours and the activity of released LDH was measured. As a positive control, cells were treated with a medium containing 1% of Triton X-100. Results are represented as a percent of the control with the mean and S.D. (n = 4).

Fig. 5. In vitro cell damage produced by SWCNTs-(KFKA)₇ with NIR irradiation.

Cell damage induced by the photothermal effect of SWCNTs with NIR irradiation was evaluated by fluorescence microscopy (A-H). Colon26 (A-D) and HepG2 cells (E-H) were exposed to SWCNTs-(KFKA)₇ for 2 hours and irradiated with an 808 nm NIR laser at 1.2 W (6 W/cm²) for 3 min. The cells were then stained with Live-Dye fluorescing green and propidium iodide fluorescing red. Photographs are shown for non-treated cells (A, E), cells treated with NIR radiation alone (B, F), with SWCNTs-(KFKA)₇ alone (C, G), and with SWCNTs-(KFKA)₇ and NIR laser (D, H). Confocal micrographs of colon26 (I) and HepG2 (J) cells having SWCNTs-(KFKA)₇ with NIR irradiation are shown by merging green and red fluorescence on bright field images.

Fig. 6. Flow cytometry evaluation of cell damage produced by SWCNTs-(KFKA)₇ with NIR irradiation.

Numbers of dead cells stained by red fluorescence were shown as percentages against total cell numbers. Colon26 (A-D) and HepG2 cells (E-H) were exposed

to SWCNTs-(KFKA)₇ for 2 hours and irradiated with an 808 nm NIR laser at 1.2 W (6 W/cm²) for 3 min. FACS patterns for non-treated cells (A, E), cells treated with NIR radiation alone (B, F), with SWCNTs-(KFKA)₇ alone (C, G), and with SWCNTs-(KFKA)₇ and NIR laser (D, H) are shown.

Fig. 7. Temperature elevation induced by NIR laser irradiation in colon26 tumors implanted subcutaneously in mice.

(A) Temperature increase in the tumor tissue given NIR laser irradiation without SWCNTs-(KFKA)₇. (B) Temperature increase in the tumor tissue injected with SWCNTs-(KFKA)₇ and given NIR irradiation.

Fig. 8. Growth inhibition of colon26 cells by photothermal therapy with SWCNTs-(KFKA)₇ and NIR irradiation.

Colon26 cells were implanted in BALB/c mice and on the eleventh day after implantation the SWCNTs-(KFKA)₇ suspension was injected into the tumor. The doses of SWCNTs were 10 µg (squares), 5 µg (diamonds), 1 µg (triangles), and 0 µg (circles). The filled and open symbols represent the groups treated with and without NIR, respectively. Results are shown as the means and S.D. (n = 5).

Fig. 9. Localization of SWCNTs-(KFKA)₇ after intratumoral injection.

Localization of SWCNTs-(KFKA)₇ composite in the tumor tissue was examined at 24 (A) and 72 hours (B) after injection. The 5 µm-section of the tumor tissue was stained with H&E and observed by photomicrography. SWCNTs-(KFKA)₇ was mostly deposited along the spaces made by needle insertion and pour of injection solution surrounded by dotted line.

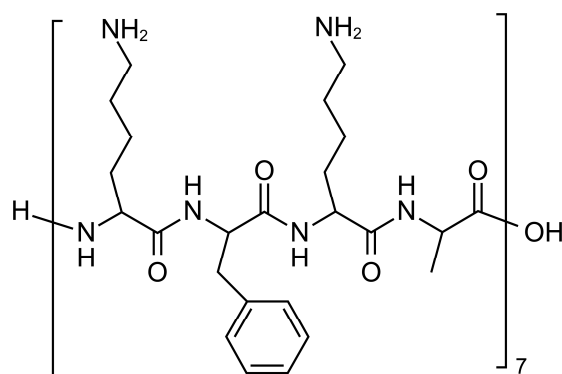


Fig. 1. Chemical structure of (KFKA)₇ peptide.

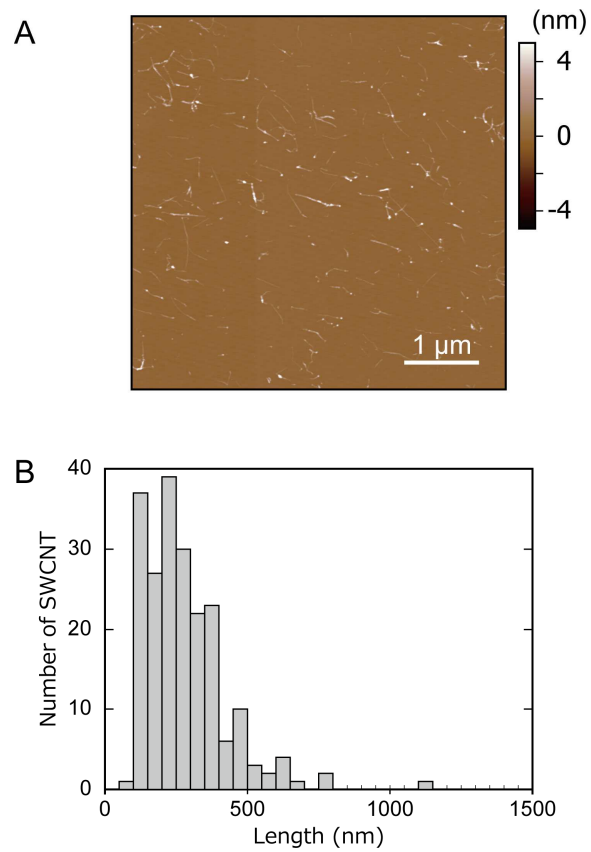


Fig. 2. AFM image (A) and size distribution (B) for SWCNTs-(KFKA)₇ in solution.

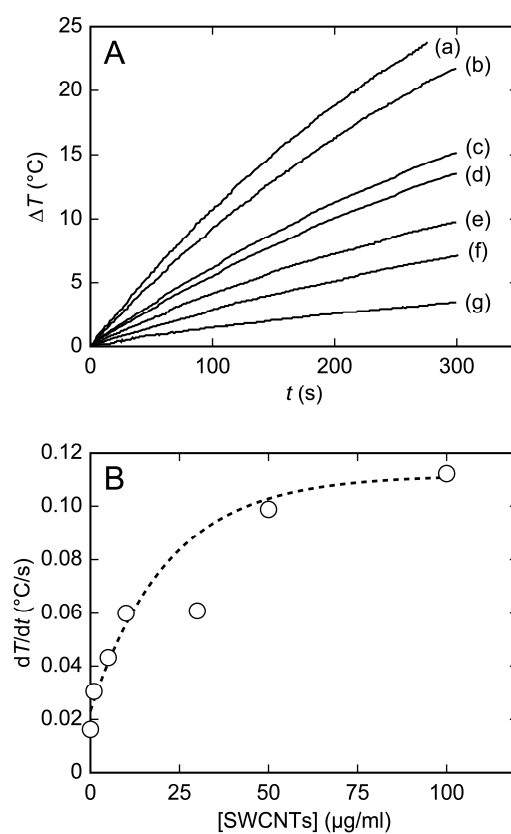


Fig. 3. Heat generation of SWCNTs-(KFKA)₇ with NIR irradiation.

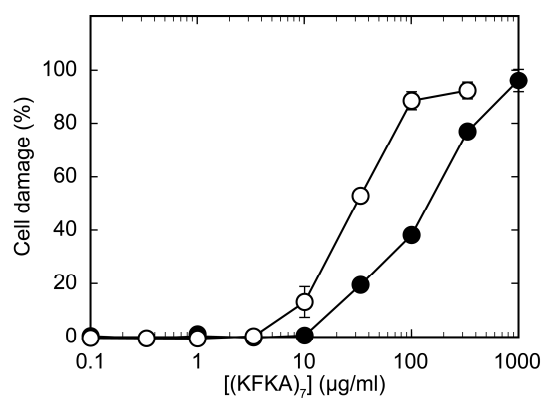


Fig. 4. Cytotoxicity of (KFKA)₇ peptide.

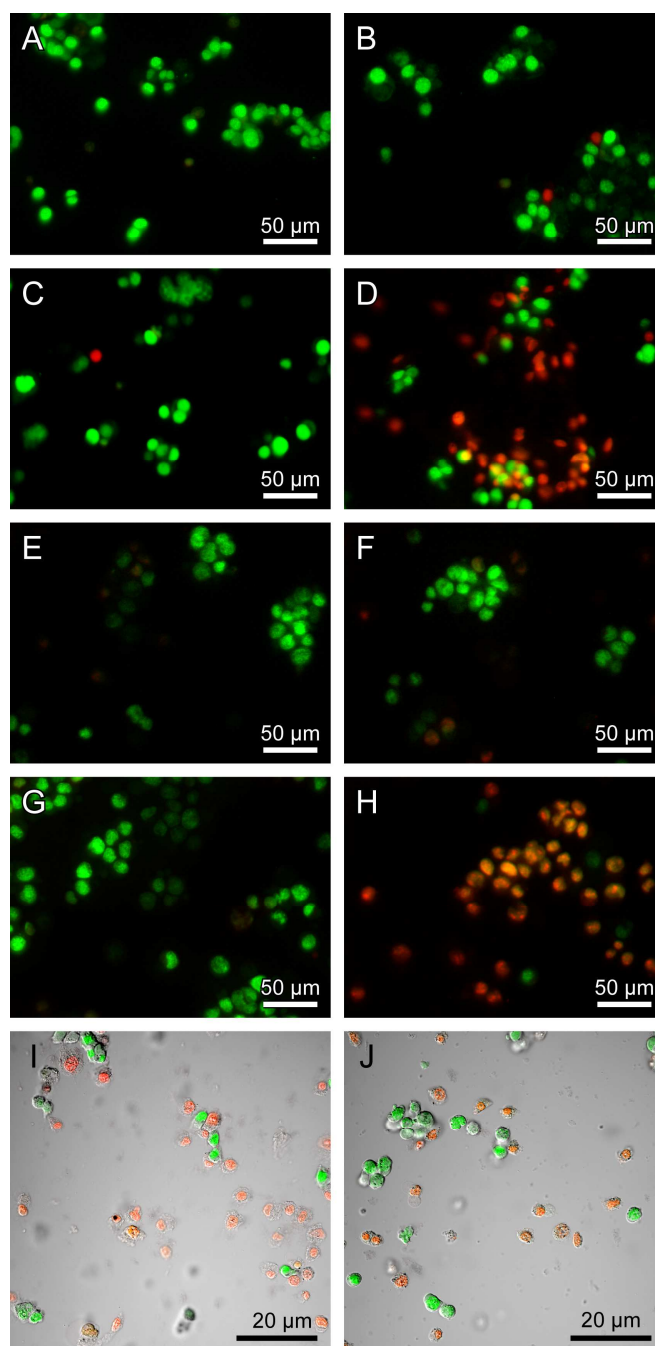


Fig. 5. In vitro cell damage produced by SWCNTs-(KFKA)₇ with NIR irradiation.

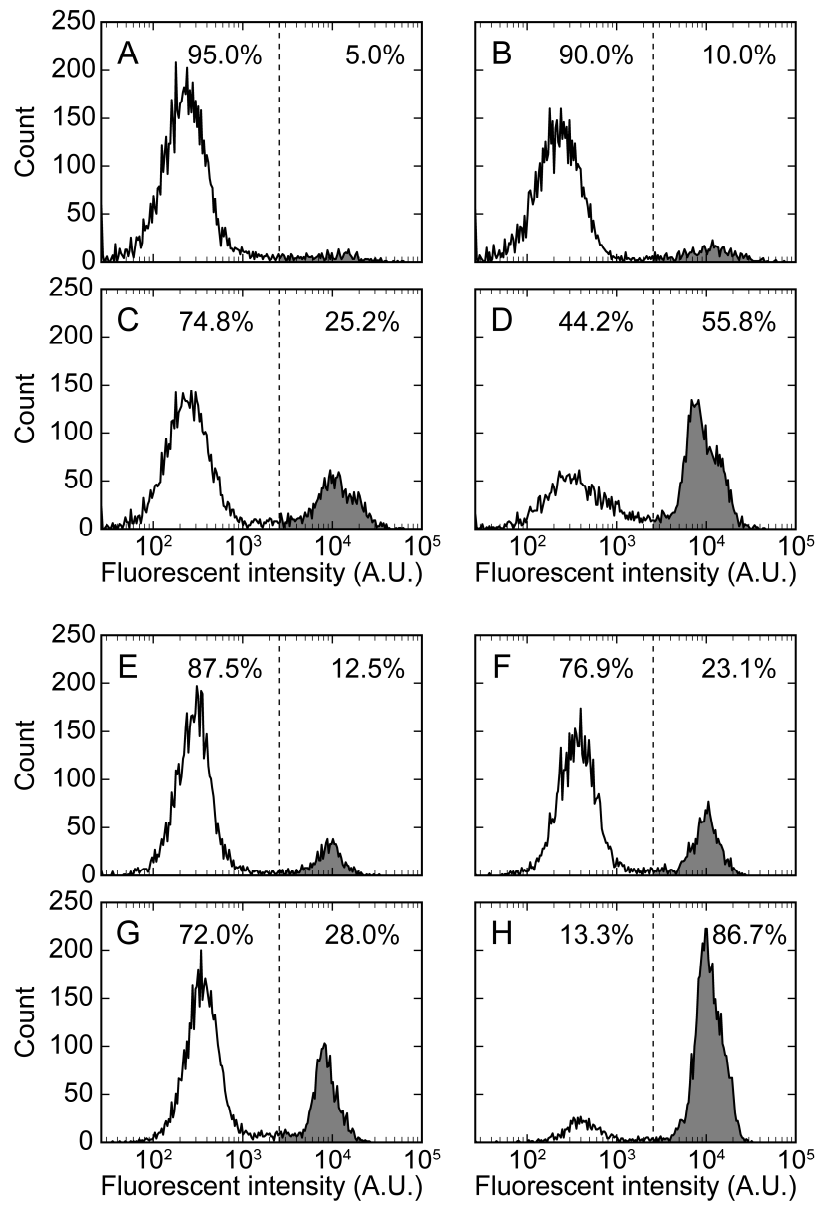


Fig. 6. Flow cytometry evaluation of cell damage produced by SWCNTs-(KFKA)₇ with NIR irradiation.

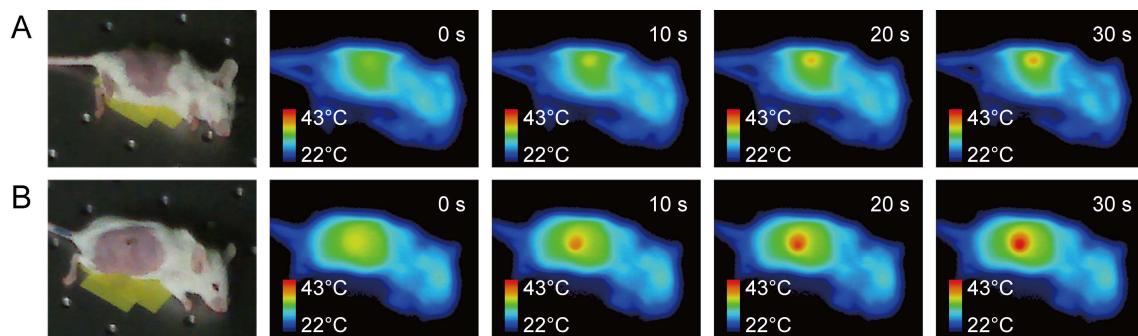


Fig. 7. Temperature elevation induced by NIR laser irradiation in colon26 tumors implanted subcutaneously in mice.

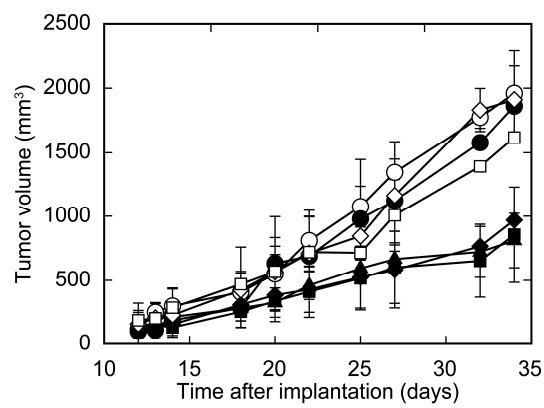


Fig. 8. Growth inhibition of colon26 cells by photothermal therapy with SWCNTs-(KFKA)₇ and NIR irradiation.

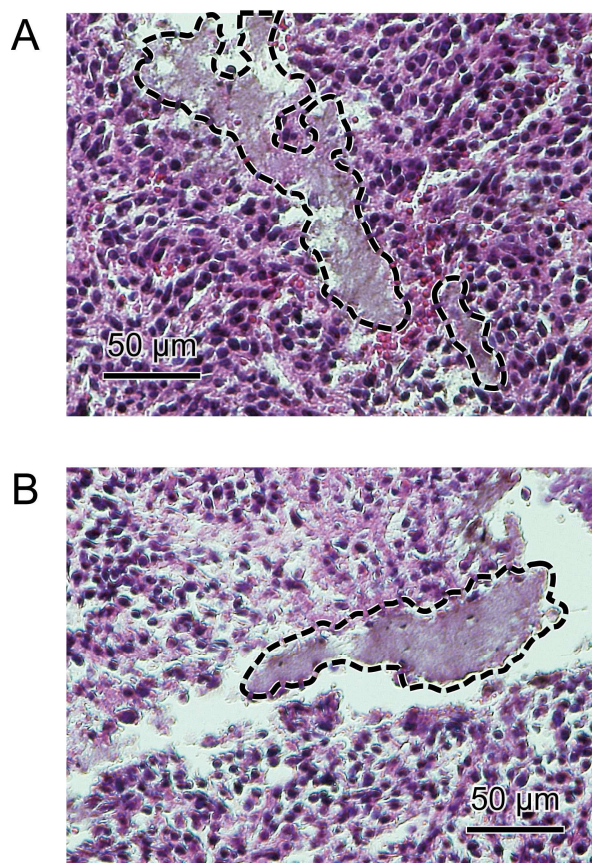


Fig. 9. Localization of SWCNTs-(KFKA)₇ after intratumoral injection.